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HIGHLY CONSERVED ASPARTATE 68, TRYPTOPHANE 73 AND GLYCINE 109 IN THE N-TERMINAL EXTRACELLULAR DOMAIN OF THE HUMAN VIP RECEPTOR ARE ESSENTIAL FOR ITS ABILITY TO BIND VIP

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The human VIP receptor belongs to a subfamily of G protein-coupled receptors that includes secretin, glucagon and several other receptors. We have used site-directed mutagenesis to investigate the requirement of highly conserved aspartate 68 (mutant D68G), tryptophane 73 (mutant W73G), proline 87 (mutant P87G), glycine 109 (mutant Δ109) and tryptophane 110 (mutant W110G) for the ability of the human VIP receptor to bind VIP. After transfection of mutated cDNAs in Cos-7 cells, it appeared that mutants G87P and W110G bound VIP with the same dissociation constant as the wild type receptor whereas mutants W73G, P87G and Δ109 did not bind VIP. Since all mutated receptor proteins were synthesized by Cos-7 cells (Western blot) and expressed at the plasma membrane level (immunofluorescence studies), it is concluded that the N-terminal extracellular domain of the human VIP receptor contains highly conserved amino acid residues which are essential for its intrinsic binding activity.

The human vasoactive intestinal peptide (VIP) receptor cDNA has been recently cloned from a jejunal epithelial cell cDNA library (1). It encodes a protein consisting of 460 amino acids with seven putative transmembrane domains like other G protein-coupled receptors (1). Alignment of the amino acid sequence of the human VIP receptor with that of other G protein-coupled receptors revealed less than 20% homology with the exception of receptors for pituitary adenylate cyclase activating polypeptide (type 1), secretin, growth hormone-releasing factor, gastric inhibitory polypeptide, glucagon, glucagon-like peptide 1, parathyroid hormone and calcitonin for which homologies range between 54 and 29 % (reviewed in 2). These receptors constitute a subfamily of the G protein-coupled receptor superfamily thereby (3). Among several common properties, they have a large N-terminal extracellular domain (> 120 amino acid residues) which contains highly conserved amino acids, including seven cystein residues, three potential N-linked glycosylation sites and five

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other residues. These amino acids are also conserved in the rat VIP receptor (4) and in a VIP type 2 receptor cloned recently from a rat pituitary gland cDNA library (5).

Recent studies from our group explored the role of conserved cystein residues[2] as well as potential N-glycosylation sites[3] in the N-terminal domain of the human VIP receptor. In this study, we have used site-directed mutagenesis to investigate the requirement of the highly conserved aspartate 68, tryptophane 73, proline 87, glycine 109 and tryptophane 110 for the ability of the human VIP receptor to bind VIP.

Material and Methods

Site-directed mutagenesis. The 1.4 Kb EcoRI fragment containing the entire coding sequence of the human intestinal VIP receptor (1) was subcloned into the p-Select vector (Promega, USA) and single strand was produced according to the manufacturer's procedure. VIP receptor mutants were generated by oligonucleotide-directed mutagenesis. Identification of desired mutations was confirmed by sequencing. Inserts encoding mutant sequences were subcloned into the eucaryote expression vector pcDNAI (In Vitrogen, UK). Wild and mutant receptors were tagged at the C-terminus with a marker dodecapeptide (Tag) as described (1).

Transfection of Cos-7 cells. Transfection of Cos-7 cells with mutant or wild receptor was performed by electroporation. Briefly, 4.10^6 cells were transfected with 20 µg purified plasmid together with 20 µg sperm salmon DNA used as carrier. After 48h, transfected Cos-7 cells were washed twice with cold phosphate-buffered saline, then harversted with a rubber policeman and collected by centrifugation at 3,000 g. Cell homogenates were prepared as previously described (1).

Ligand binding assay. The functional properties of wild type and mutant VIP receptors were analyzed by [^{125}I]-VIP binding to transfected cell homogenates as described (1). Specific binding was calculated as the difference between the amount of [^{125}I]-VIP bound in the absence and the presence of 1µM unlabeled VIP. Binding data were analyzed using the LIGAND computer software (6).

Western blot. 50 µg membrane proteins were runned onto 9% SDS-polyacrylamide gel and then transferred to nitrocellulose sheets as described (7). The nitrocellulose sheets were preincubated for 2h in 50 mM Tris buffer, pH8, containing 2 mM CaCl2, 80 mM NaCl, 5% (w/v) dry milk and 0.2% (v/v) NP40 as previously described (7), then incubated with the anti-Tag antibody 9E10, and after 3 successive washings revealed with [^{125}I]-labeled F(ab')2 sheep anti-mouse Ig. Blots were washed, dried and autoradiographied for 2 days as described (7).

Immunofluorescence studies. Immunofluorescence studies of tagged wild type and mutant receptors after transfection of Cos-7 cells were performed as previously reported (1).

Results and Discussion

Figure 1 shows multiple alignment of the VIP / secretin subfamily of G protein-coupled receptors in a N-terminal extracellular part of their amino acid

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[2] Gaudin, P., Couvineau, A., Maoret, J., Rouyer-Fessard, C., and Laburthe, M., in preparation.

[3] Fabre, C., Couvineau, A., Gaudin, P., Maoret, J., and Laburthe, M., in preparation.

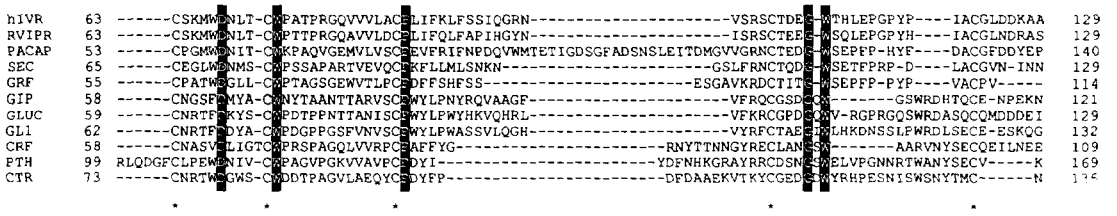

Figure 1. Amino acid sequence alignment of region 63-129 of the N-terminal extracellular domain of the human VIP receptor (hIVR) with the corresponding region in other members of the VIP receptor subfamily of G protein-coupled receptors,i.e., rat VIP receptor (RVIPR), rat PACAP (type I) receptor (PACAP), rat secretin receptor (SEC), human GRF receptor (GRF), rat GIP receptor (GIP) (13), rat glucagon receptor (GLUC), rat GLP-1 receptor (GL1), human CRF receptor (CRF) (14), opossum PTH receptor (PTH) and porcine calcitonin receptor (CTR). Black boxes represent the five highly conserved amino acids D^{68}, W^{73}, P^{87}, G^{109} and W^{110} and stars indicate the highly conserved cysteine residues in this region. See ref. 2 for a review regarding the complete amino acid sequences of these receptors.

sequences. It appears clearly that D^{68}, W^{73}, P^{87}, G^{109} and W^{110} in the human VIP receptor are highly conserved in all other receptors of this subfamily. In order to determine the role of these five residues in the ability of the human VIP receptor to bind VIP, five mutants were first constructed by site-directed mutagenesis : D68G, W73G, P87G, and W110G in which the wild amino acid has been replaced by a glycine, and Δ109 where the glycine residue was deleted. After transfection of the mutant cDNAs in Cos-7 cells, specific [^{125}I]-VIP binding to cell membranes was determined (Table I). It appears that the mutants G87P and W110G bound VIP with the same dissociation constant as the wild type receptor, indicating that P^{87} and W^{110} residues were not involved in the affinity of the human VIP receptor for VIP. In contrast, when mutants D68G, W73G, Δ109 were transfected in Cos-7 cells no specific [^{125}I]-VIP binding could be detected (Table I). In order to verify that deletion of G^{109} did not induce conformational change by modification of the chain length, substitution mutation was carried out by replacing the glycine residue by aspartic acid or tryptophan residues (G109D, G109W). After transfection of these two mutant cDNAs no specific [^{125}I]-VIP binding could be detected either (Table I).

The absence of [^{125}I]-VIP binding observed with mutants D68G, W73G and Δ109 could be due to the fact the mutated receptor proteins have no intrinsic binding activity or to alteration in their synthesis and/or plasma membrane addressing. In order to test these two latter hypotheses, we took advantage of the presence of a Tag at the C-terminus of the wild and mutated receptor proteins. Immunoblot analysis of membrane proteins using monoclonal anti-Tag antibodies revealed the presence of a major 69 kDa band after transfection of the wild receptor cDNA (Figure 2). This band corresponds to the glycosylated human VIP receptor protein as previously described (8). As a control, when Cos-7 cells were not

Table I : Scatchard analysis of ^{125}I-VIP binding to wild type (wt) and mutated human VIP receptors

	Kd (nM)	Bmax (pmoles/ mg of prot.)
wt	0.59±0.12	6.43±0.88
D68G	ND	ND
W73G	ND	ND
P87G	0.60±0.20	0.73±0.12
Δ109	ND	ND
G109D	ND	ND
G109W	ND	ND
W110G	0.62±0.10	5.19±1.44

The binding assay was performed on homogenates from transfected Cos-7 cells. Each value is the mean ± S.E. of three experiments.
ND, binding Not Detectable.

transfected with the human VIP receptor cDNA no band was detected (Figure 2). Western blot studies using membrane proteins of Cos-7 cells transfected with mutant cDNAs also revealed 69 kDa bands whose intensities were similar to that observed with the wild receptor (Figure 2). It is therefore likely that all receptor mutants are synthesized by Cos-7 cells in a manner qualitatively and quantitatively similar to that of the wild receptor. Immunofluorescence studies using the anti-Tag antibodies were then conducted on Cos-7 cells transfected with mutant and wild

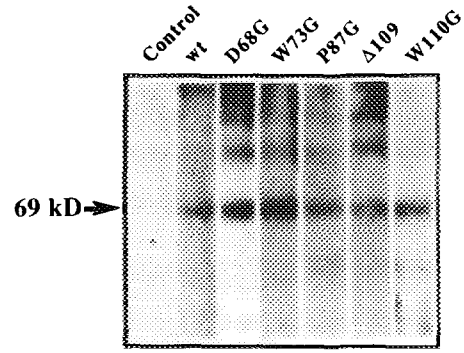

Figure 2. Western blot of wild type (wt) or mutated human VIP receptors expressed in Cos-7 cells. Homogenates from Cos-7 cells transfected without (Control) or with wild type or mutated cDNAs were separated on SDS-PAGE, transfered on nitrocellulose and immunodetected using the anti-Tag antibody.

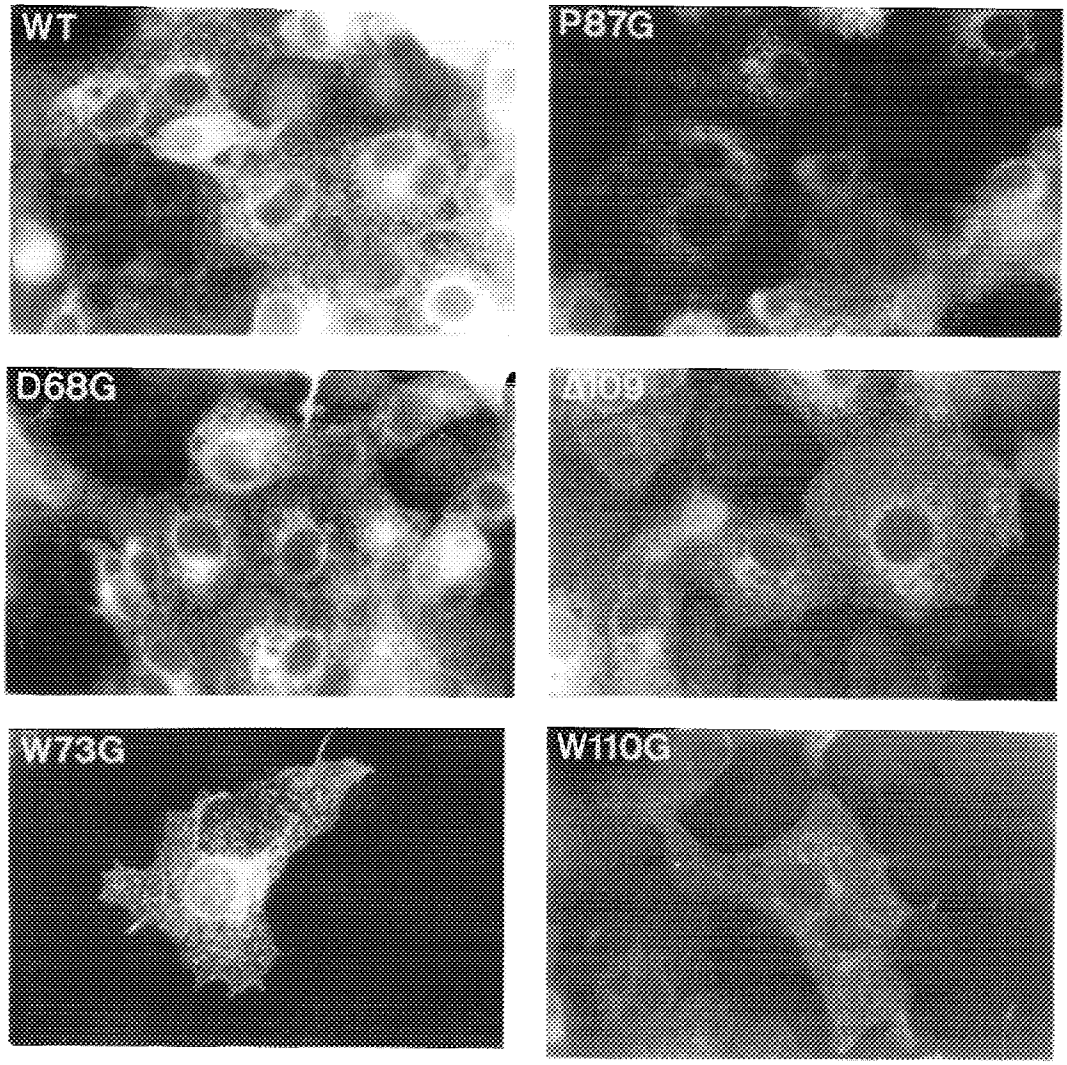

Figure 3. Immunofluorescence detection of the tagged wild type (wt) or mutated human VIP receptors on transfected Cos-7 cells. The tagged proteins (wt or mutated) are expressed at the plasma membrane.

receptor cDNAs. As shown in Figure 3, the wild receptor as well as the D68G, W73G, P87G, Δ109 and W110G VIP receptor mutants could be detected at the plasma membrane of transfected cells. Taken together these results suggest that the D^{68}, W^{73} and G^{109} residues play a key role in the intrinsic binding activity of the human VIP receptor since point mutation of these residues resulted in the absence of [^{125}I]-VIP binding although mutated receptor proteins were normally synthesized and addressed at the plasma membrane level.

The present data demonstrate that the N-terminal extracellular domain of the human VIP receptor contains at least three residues aspartate 63, tryptophane 73 and

glycine 109 that are essential for its ability to bind VIP. The fact that these residues are conserved in all receptors in the subfamily of G protein-coupled receptors which includes the VIP receptor, suggests that those three amino acids are also crucial in the ability of receptors of this subfamily to bind their respective natural peptide ligands. This cannot be definitively assessed yet in the absence of data regarding all theses receptors, many of which have been recently cloned (reviewed in 2). However, recent data regarding the phenotype *little* mouse (*lit/lit*) showed that it is associated with the existence of a mutated GRF receptor at aspartate 60 (aspartate → glycine) resulting in the complete loss of binding of GRF (9). This aspartate is aligned with the crucial aspartate 68 in the human VIP receptor (see Table I) supporting that this residue plays an essential role in peptide ligand binding in the subfamily of VIP / GRF receptors.

This work evidences that the N-terminal extracellular domain of the human VIP receptor contains amino acid residues that are essential for VIP binding. This was further supported by two other sets of data from our group : 1) Some of the highly conserved cystein residues in this domain are also essential for the ability of the receptor to bind VIP [1], suggesting that they maintain a functional tertiary structure of the N-terminal tail (2); 2) When the N-terminal domain was deleted by the construction of a cDNA coding for a truncated receptor protein where the initiation codon begins at the T^{144} residue, no [^{125}I]-VIP binding could be detected after transfection of Cos-7 cells (not shown). Altogether these preliminary observations already support that the human VIP receptor and most probably other receptors of its subfamily are quite different from adrenergic receptors such as the β_2-adrenergic receptor where deletion of most of the extracellular residues did not affect the binding of biogenic amines (10), in consonance with the existence of a binding pocket within the transmembrane domains (10). In that respect, the human VIP receptor resembles most other G protein-coupled peptide receptors for which extracellular domains play an important role in peptide binding (10). However, the structure stabilized by S-S bonds[2] may indicate that this region plays a more important role than in most other G protein-coupled peptide receptors. This has been already demonstrated for glycoprotein hormone receptors such as the lutropin receptor whose long N-terminal region (338 amino acids), when expressed in the absence of the transmembrane domains, exhibits high affinity and specificity for luteinizing hormone (11). Whether VIP receptors and other members of its subfamily of G protein-coupled peptide receptors exhibit the same properties is currently under investigation in our laboratory. Anyhow, following on the purification of VIP receptors from rat liver in 1990 (12), their recent cloning from several tissues and species (1,3,4) has provided a tremendous acceleration in the pace at which the structure-function relationship of VIP receptors is unraveled.

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